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Bioactivity and Volatile Profiling of *Azadirachta indica* Leaves for the Management of Maize Weevil, *Sitophilus zeamais* (Motsch.) Infestations

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Abstract

Neem (*Azadirachta indica* A. juss), is known to possess a wide range of pharmacological properties and is thus commercially exploitable. Apart from its medicinal potential, a considerable progress has been achieved regarding biological potential and chemical composition of the leaves which is an ever-increasing interest to the scientific community. During this study, biological phenomena and secondary metabolite composition of *A. indica* leaves were examined in the management of *Sitophilus zeamais* on stored maize. Insecticidal and repellent potential of *A. indica* leaf powders were evaluated in both contact and fumigant forms. Phytochemical screening of 11 phyto constituents was performed following the standard procedures for n-hexane, dichloromethane, ethyl acetate, methanol and aqueous leaf extracts. Volatile profile of *A. indica* leaves was characterized by employing headspace-solid-phase micro extraction coupled with gas chromatography-mass spectrometry (HS-SPME/GC-MS). Over 60% weevil repellency was recorded at doses above 23.33%, whereas 100% and 67% contact and fumigation mortalities were observed respectively, 9 days after treatment at the dose of 33.33% and the respected LD_{50} values were 1.56 g and 4.48 g. Thirty two volatile compounds were identified in three distinct chemical classes (Monoterpenoid, sesquiterpenoid and purine nucleosides). γ-Elemene (24.06%), 3,7 (11)-eudesmadiene (6.83%), caryophyllene (6.40%), and 10s,11s-himachala-3(12),4-diene (6.36%) were the major constituents of neem leaf volatiles, followed by other compounds present in less than 4% which might be responsible for varied biological activities observed. Thus the odour impact of the bioassay-guided study clearly implies that *A. indica* leaves can be harnessed against *S. zeamais* infestations.

Keywords: Azadirachta indica, Sitophilus zeamais, headspace-solid-phase micro-extraction, insecticidal activity, repellency

1. Introduction

Maize (*Zea mays*) is a versatile crop among the three most important cereal crops of the world, the other two being wheat and rice. It possesses a prominent genetic diversity and is grown over a range of agro climatic zone (Ministry of Environment, Forests & Climate Change, Government of India and Department of Biotechnology, 2011). In Sri Lanka, the maize cultivation has become a highly commercialized venture during the last several years (Department of Agriculture, 2013). Though, the factors restricting the maize production are also diverse, the most threatening being the insect attack during the storage (Ortega, 1987). More recently, in every respect of the world, attention of the researchers has been paid towards the exploitation of plant products as stored grain protectants against insect pests due to the increasing public concern over the level of insecticidal residues in food (Dubey et al., 2008).

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IPM programs have demonstrated that current levels of insecticidal use in many circumstances are not necessary and, frequently, are even counter-productive (Khater, 2012). Many farmers in Sri Lanka may not be able to afford synthetic insecticides and even when those are affordable to growers through government subsidies, limited literacy and lack of protective equipment make way to thousands of accidental poisonings annually (Isman, 2006).

The plant kingdom offers a rich source for a wide array of structural biodiversity of natural secondary metabolites (El-Wakeil, 2013). Insect-plant interactions have been studied for many years, but an extraordinarily discerning account on these complex co-adaptive relationships could provide a basis for using plant derived chemicals in green approaches for a better management of insect pests inhabiting stored grains (Coats et al., 1991). They can play a huge role in developing countries like ours as a new class of eco-friendly and biodegradable products for controlling pests. Also, they offer unique and challenging opportunities for exploration, development and commercialization of their own botanicals. There is a wide scope for the use of plant-based insecticides in the integrated management of stored insect pests because botanicals may help in preventing the dumping of tons of insecticides on earth (Khater, 2012; Adeyemi and Mohammed, 2014). Moreover, the advances in chemical and biological technologies combined with increasing need and environmental pressure, greatly increase the interest in the development of plant products as green insecticides (Khater, 2012).

One such phenomenal source of green insecticide is the neem tree (*Azadirachta indica* A. Juss; Meliaceae). The components taken from this evergreen tree (leaves, seeds and bark) have demonstrated an unusual biological effectiveness against a wide spectrum of insect pests (Zeringue and Bhatnagar, 1994). Although many reviews have been published on the biological activity of other neem components, chemical basis underlying the bioactivity of neem leaves has not received much attention in the suppression of insect pest infestations.

As a step in the path towards the exploration and continuation of the studies on neem tree components, the impetus of this research was to assess the insecticidal activity of neem leaves against the maize weevil (*Sitophiuls zeamais*, Motsch) which is a serious cosmopolitan field-to-store insect pest of maize and other cereal grains in tropical and subtropical regions of the world. In order to obtain a more realistic picture of the entire range of volatile organic compounds emitted by neem leaves which detected by maize weevils that responded to leaf organic volatiles, headspace solid-phase microextraction (HS-SPME) technique was also employed in the present study.

2. Materials and Methods

A series of experimental designs were conducted at the agricultural insect pest management laboratory of Department of Zoology, Faculty of Applied Sciences, University of Sri Jayewardenepura, Sri Lanka from January to July 2016. The relative humidity and ambient temperature during the experimental period were 84±2% RH and 29±2^{o} C respectively. All the assessments carried out in this study were replicated five times.

2.1 Collection of plant material

Fresh, mature and healthy leaves of *Azadirachta indica* were collected from Gangodawila area. These were ground into a fine powder using a domestic electric grinder (Multinational®, 2101, India) and were packed in glass containers with tight lids and stored in a refrigerator at 4^{o} C prior to use in the bioassays.

2.2 Maintenance of Sitophilus zeamais cultures

Sitophilus zeamais used for the study were obtained from infested stock of maize in the local market and then reared on un-infested maize grains in glass jars covered with muslin cloth held in place with rubber bands for the passage of air and prevention of the weevil escape. Unsexed adult weevils of 3-7 days old were used for all experiments.

2.3 Sample preparation for phytochemical screening

The coarse powders were subjected to successive extraction in various solvents such as water, n-hexane, ethyl acetate, methanol and dichloromethane using Soxhlet apparatus. The collected extracts were then taken up for further investigations.

2.4 Contact repellent effect

The freshly powdered plant leaves were admixed with clean and un-infested maize grains at 5 doses (3.33%, 10%, 16.67%, 23.33%, and 33.33% w/w) per thirty grams (30 g) in separate small plastic cups (height 8 cm, diameter 7.5 cm). One week old, 20 adult weevils were introduced into each cup. The top ¼ height of the small plastic cup was perforated using a soldering gun (220V/240V, 40W, China). These holes were made to allow the weevils to escape from the plastic cup if they are repelled by the plant powders. This small plastic cup was placed inside a large plastic bottle (height 15 cm, diameter 7.5 cm) to trap the weevils moving out through the holes. Before the onset of each experiment, the holes were covered with a sticky tape for 10 minutes to let the introduced maize weevils settle down inside the plastic cup. The bio apparatus was then covered with muslin cloth held in place with rubber bands to allow ventilation of weevils. The same bio apparatus filled only with 30g of rice without leaf powder was considered as the control. The number of repelled insects in the large plastic bottle was counted 1 hour after their introduction to estimate maize weevil repellency.

2.5 Fumigation repellent effect

The bio apparatus used was somewhat similar to the setup in the contact repellency bioassay but with some alterations. The bottom of the small plastic cup was removed and replaced by a nylon cloth which was then fitted with a small plastic container (height 4 cm, diameter 5 cm) to place the leaf powders inside the latter. This adjustment allowed the vapor of leaf powders inside the container to pass through the cloth and reach the weevils. Leaf powder was then put in the plastic container at the 5 rates of 3.33%, 10%, 16.67%, 23.33%, and 33.33% w/w, and 30 g of maize grains were placed in the small plastic cups. One week old, 20 adult weevils were introduced into the small plastic cups. Grains in the control test contained no leaf powders. Number of repelled weevils in the large plastic bottle was counted one hour after introduction.

2.6 Contact toxic effect

Contact toxicity was assayed by admixing 30 g of uninfested maize grains with powdered leaves of plants at the doses of 3.33%, 10%, 16.67%, 23.33%, and 33.33% w/w in the plastic containers (height 8 cm, diameter 7.5 cm). One week old, 20 adult weevils were introduced into each cup. The containers were covered with muslin cloth held firmly with rubber bands to prevent the escape of the weevils and to ensure adequate aeration. Maize grains with no plant powders were included to serve as the control. The contact mortality of the weevils was recorded at 24 hour intervals up to 10 days of weevil exposure.

2.7 Fumigation toxic effect

The bio apparatus for the fumigation toxicity test was consisted of a small plastic container (height 4 cm, diameter 5cm) attached to a plastic cup (height 8 cm, diameter 7.5 cm). The bottom of the plastic

cup was removed and replaced by a nylon cloth to allow the vapor of plant powders in the container to pass through the cloth and reach the weevils. Leaf powders were put in the separate plastic containers at the doses of 3.33%, 10%, 16.67%, 23.33%, and 33.33% w/w while 30 g of clean and uninfested maize grains were placed in the plastic cups. One week old, 20 adult weevils were introduced into each plastic cup. The bio apparatus was covered with muslin cloth held in place with rubber bands. Bio apparatus without the leaf powders was kept as the positive control. Fumigant mortality of the maize weevils was evaluated daily for 10 days of weevil exposure**.**

2.8 Preliminary qualitative phytochemical screening

The crude n-hexane, dichloromethane, ethyl acetate, methanol and aqueous leaf extracts were subjected to phytochemical screening following the standard methods as described by Harborne (1998) and Hsu et al. (1981).

2.9 Isolation of volatile organic compounds (VOCs)

SPME Fibers

SPME holder and fiber assemblies for manual sampling were provided from Agillent Technologies (Palo Alto, CA) and used without modification. The fiber coatings assayed were as follows: polydimethylsiloxane (PDMS 30 µm), polyacrylate (PA 85 µm) (Table 1). Before measurements, the fiber was conditioned in the injector for 30 minutes at 250^{o} C with split vent open to fully remove any contaminant that might cause high baseline noise and large ghost peaks. Then the fiber was repeatedly injected into the GC until interfering peaks disappeared. During this desorption process the GC column oven temperature was maintained at 250^{o} C.

Table 1: SPME fibers used during the microextraction technique.

Fiber Type	Acronym	Full Name	Volume of Coating (mm^3)
Medium-polar	PDMS 30	Polydimethylsiloxane, 30µm	0.132
Polar	PA 85	Polyacrylate, 85 µm	0.521

Headspace solid-phase microextraction procedure

The HS-SPME extraction after optimization was performed by placing 0.3 g of freshly ground neem leaf powder in 12 ml crimp-top headspace vial (diameter 2 cm, height 6.7 cm), capped with porous poly-tetrafluoroethylene (PTFE) silicon rubber septum. The sample in the headspace vial was heated by supporting them with a clamp in a hot water bath (60^{o} C). After 10 minutes, needle of the SPME device was pierced the septum of the vial and the fiber was immersed to the headspace of the sample for 30 minutes, 1 cm above the leaf powder, which was kept at 60^{o} C. After extraction, the fiber was inserted into the hot injector of the GC systems for analysis.

Gas chromatography–mass spectroscopy (GC-MS) analysis conditions

Chromatographic analysis was performed using an Agilent Technologies 7890A gas chromatograph (Palo Alto, CA) equipped with an Agilent Technologies 5975C inert XL EI/CI mass selective detector. A DB-5MS fused silica capillary column of 30 m×0.25 mm i.d.×0.25 µm film thickness (J & W Scientific, Folsom, CA) was used. Helium was used as the carrier gas at a flow rate of 1ml/min and detector gases were hydrogen and air. The temperature was programmed as follows: initial oven temperature was 40^{o} C for 3minutes, and then was increased at 10^{o} C/min up to 280^{o} C, where it was held for 3 minutes and maintained constant for 30 minutes. The injection port was in the splitless mode. SPME fiber was introduced in the injector port, held at 250^{o} C for chromatographic analysis and remained in the inlet for 30 minutes. Identification of components in the sample was based on the chromatographic

criteria (retention times) and MS spectral library at the Chemistry Department in University of Sri Jayewardenepura, Sri Lanka.

2.10 Analysis of data

All data were subjected to one-way analysis of variance (ANOVA) using the "Minitab", version 14.0. Tukey's multiple comparison test was used to separate mean values of the experiments, where significant differences existed ($p<0.05$). Probit analysis was used to estimate LC_{50} values to determine the lethal concentrations needed to kill 50% of weevils.

3.0 Results and Discussion

3.1 Contact Insecticidal Effect

The contact toxic effect of neem leaf powders on the survival of maize weevil adults are presented in Table 2. Tested leaf powders of *A. indica* significantly ($P<0.05$) reduced the longevity of adults on treated maize grains apart from the control which gave no weevil mortalities. At highest dose of 33.33 % w/w, all the leaf powders produced contact weevil mortalities ranging from 34–100% and 7-52% at the lowest dosage (3.33% w/w) within 1-10 day time period. Accordingly, *A. indica* leaf powders showed its superiority in suppressing *Sitophilus zeamais* populations evoking 100% contact mortality, at the highest dosage after 9 days of weevil exposure. Moreover, as the exposure time proceeds, there was a progressive increase in the insecticidal potential of the botanical to the maize weevils, resulting in considerably high mortality of *S. zeamais*.

According to some previous literature, neem leaves were sufficient enough in protecting cacao beans and jola seeds from insects and found to exhibit insecticidal activities over *Ephestia cautella.* Additionally, these leaves have been placed in 6-8 cm layers which then protected the grains from *Sitophilus oryzae*, *Sitotroga cerealella* and *Rizopertha dominica*. It was also reported that dried neem leaves have been admixed with stored paddy or a thick layer of 20-30 cm leaves between the bags and floor to protect stored paddy from insects Moreover, neem leaves are evidenced in protecting rice and wheat grains stored in gunny bags against wide array of grain boring insects. Furthermore, neem leaf powder displayed toxicity to *Callosobruchus chinensis* and absolute grain protection on *Rizopertha dominica* for 6 months in storage while reducing adult emergence of *Corcyra cephalonica* by 52-56% and larval mortality by 40-45% in sorghum (Prakash and Rao, 1997).

The neem leaf powder on application usually covers the testa of maize grains, serving as food poison to the adult insects (Ileke and Oni, 2011). Azadirachtin, the neem's agent of controlling insects (Rejitha et al., 2014), produces an antiperistalitic wave in the insect alimentary canal thus creating a vomiting sensation in the insect. Due to that lethal sensation, the insect does not feed on the neem treated surfaces and their ability to swallow is also blocked thus eventually leading to their death (Lokanadhan et al., 2012).

Table 2: Contact toxic effect of *A. indica* leaf powders on *S. zeamais* at 24 hour interval up to 10 days.

Time/ HAD	*Mean % Contact Mortality ± SD					
	Dose (w/w %)					
	Control	3.33	10.00	16.67	23.33	33.33
1	ND	7.00±2.74^{b}	11.00±2.74^{bc}	13.00±2.74^{c}	16.00±4.18^{c}	34.00±5.48^{d}
2	ND	9.00±2.24	16.00±4.18^{c}	24.00±2.24	30.00±0.00^{e}	48.00±2.74^{f}
3	ND	12.00±2.74^{b}	18.00±2.74^{c}	29.00±4.18^{d}	42.00±2.74^{e}	57.00±2.74^{f}
4	ND	13.00±4.47^{b}	26.00±2.24^{c}	32.00±2.74^{d}	43.00±2.74^{e}	68.00±2.24^{f}
5	ND	16.00±2.74^{b}	30.00±5.00^{c}	34.00±3.54^{c}	44.00±2.74^{d}	72.00±2.74^{e}
6	ND	23.00±2.74^{b}	34.00±4.18^{c}	38.00±2.74^{d}	46.00±2.24^{e}	79.00±2.24^{f}
7	ND	26.00±4.18^{b}	41.00±4.18^{c}	47.00±4.47^{c}	62.00±2.74^{d}	86.00±5.00^{e}
8	ND	39.00±2.24^{b}	57.00±4.47^{c}	68.00±2.74^{d}	71.00±4.18^{d}	94.00±4.18^{e}
9	ND	42.00±2.74^{b}	65.00±5.00^{c}	73.00±2.74^{d}	82.00±2.74^{e}	100.00±0.00^{f}
10	ND	52.00±4.47^{b}	72.00±2.74^{c}	76.00±4.18^{c}	84.00±4.18^{d}	100.00±0.00^{e}

*Means followed by the same letters in each row are not significantly different according to the Tukey's test at $P<0.05$; *Mean Percentage Contact Mortality±*SD* for five replicates (n = 100); HAD – Days after Treatment; ND=Not Detected

3.2 Fumigation insecticidal activity

The evaluation of fumigation toxicity of *A. indica* leaf powder against maize weevils, revealed its potential of controlling test insects as a fumigant (Table 3). Least fumigation mortality (29.00±2.24%) was observed when the maize grains were treated at 3.33% w/w of lowest dose, while the highest dose (33.33% w/w) was producing 77.00±2.74% *S. zeamais* mortality after 10 day time interval while positive control displaying no mortality. Farmers in Sri Lanka have long been used to burn neem leaves to generate smoke for fumigation against insect pests of stored paddy and pulses (Ranasinghe, 1984; Saxena, 2009). It is noted that the pungent smell of this natural fumigant not only kills insect pests but also affects them negatively by acting as feeding and oviposition deterrent, mating disruptor and growth inhibitor in the protection of stored grains (Lokanadhan et al., 2012) and would not allow the formation of resistant races of the insect which is quite common with most of the synthetic insecticides (Shukla et al., 2007). It was also recorded that neem leaf volatiles drastically reduces the hatchability of the cotton bollworm, *Earias vittella* (Prakash and Rao, 1997). Mulungu et al. (2007) and Yohannes et al. (2014) have noted that fumigation mortality occurs as a result of physical barrier effects exerting by the plant powders on insects. In that context, powders have the tendency to block spiracles of the insects thus causing asphyxiation and impair physiological processes by penetrating the insect body via respiration system ultimately leading to the death of insects (Melo et al., 2015).

Table 3: Fumigation toxic effect of *A. indica* leaf powders on *S. zeamais* at 24 hour interval up to 10 days.

*Mean % Fumigation Mortality ± SD

Time/ HAD	Dose (w/w %)					
	Control	3.33	10.00	16.67	23.33	33.33
1	ND	0.00±0.00^{a}	0.00±0.00^{a}	2.00±2.74^{a}	8.00±4.47^{b}	14.00±2.24^{c}
2	ND	0.00±0.00^{a}	0.00±0.00^{a}	5.00±5.00^{b}	10.00±3.54^{b}	21.00±4.18^{c}
3	ND	0.00±0.00^{a}	3.00±4.47^{a}	11.00±4.18^{c}	14.00±4.18^{c}	25.00±3.15^{d}
4	ND	8.00±2.74^{b}	13.00±2.74^{b}	21.00±2.24^{c}	24.00±2.24^{c}	33.00±4.47^{d}
5	ND	12.00±2.74^{b}	16.00±2.24^{b}	25.00±2.54^{c}	28.00±2.74^{c}	39.00±4.18^{d}
6	ND	13.00±2.74^{b}	18.00±4.47^{b}	30.00±0.00^{c}	32.00±2.74^{c}	47.00±2.74^{d}
7	ND	19.00±4.18^{b}	27.00±4.47^{c}	35.00±3.54^{d}	40.00±3.54^{d}	49.00±2.24^{e}
8	ND	23.00±2.74^{b}	32.00±4.47^{c}	37.00±4.47^{c}	43.00±2.74^{d}	54.00±2.24^{e}
9	ND	21.00±4.18^{b}	46.00±5.48^{c}	53.00±2.74^{d}	59.00±2.24^{d}	67.00±2.74^{e}
10	ND	29.00±2.24^{b}	48.00±2.74^{c}	60.00±0.00^{d}	65.00±0.00^{e}	77.00±2.74^{f}

*Means followed by the same letters in each row are not significantly different according to the Tukey's test at $P<0.05$; *Mean Percentage Fumigation Mortality ± *SD* for five replicates (n = 100); HAD – Days after Treatment; ND=Not Detected

The leaf powders of *A. indica* exerted significant ($p<0.05$) contact and fumigation insecticidal activities against *S. zeamais* with respect to the median lethal dose (LD_{50}) values after 9 days of exposure (Table 4). However, it was observed that the fumigation treatment (4.48 g) was 3 times less active against the maize weevils than the contact treatment (1.56 g).

Table 4: Median lethal doses (LD_{50}) of *S. zeamais* due to *A. indica* leaf powders after 9 days of exposure.

Treatment	LD_{50} (g)	Confidence Interval		Slope±SE	*p* value
		Lower	Upper		
Contact	1.56	1.17	1.93	0.73±0.08	0.00
Fumigation	4.48	3.66	5.52	0.52±0.08	0.00

95% lower and upper fiducial limits are shown in parenthesis

LD_{50} – Lethal dosage that kills 50% of the population

3.3 Contact and fumigation repellent activity

Mean percentage contact and fumigation repellency of *S. zeamais* adults to maize grains treated with varying leaf powder doses of *A. indica* are presented in Figure1.

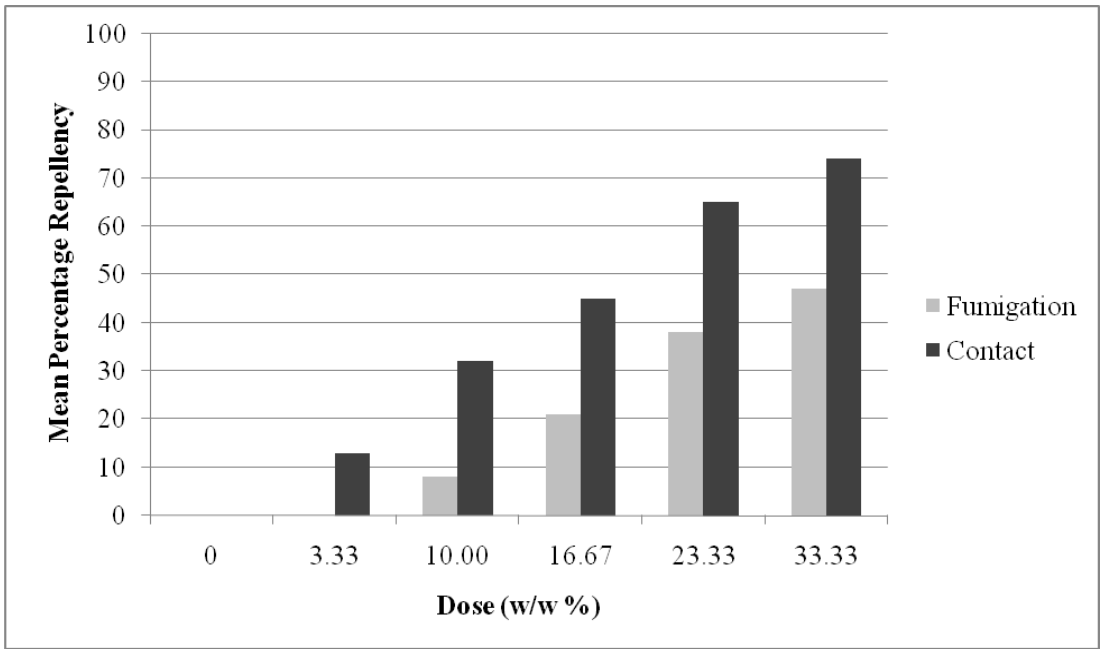

Figure 1: Contact and fumigation repellency effect of neem leaf powder on *S. zeamais* within an hour of weevil exposure.

It was evidenced that *S. zeamais* adults were more significantly susceptible to contact treatments than to the fumigation treatments with regards to repellency. Leaf powders of *A. indica* hindered the orientation of 74% and 47 % of adults in contact and fumigation treatments respectively at the highest dose of 33.33% w/w. It is noteworthy that all tested doses in both treatments produced less than 50% repellent effects on maize weevils except for 23.33% and 33.33% w/w doses in contact treatment. The relatively lower fumigation effects would be due to the low potentiality of neem leaf volatiles as fumigants which may not be enough to repel maize weevils. In Sri Lanka, chopped green leaves of *A. indica* were being kept over the heap of paddy in a container to hinder the insect orientation towards the paddy or to repel the insects from the grains (Saxena, 2009).

3.4 Preliminary qualitative phytochemical screening

Table 5 shows the list of classes of plant secondary metabolites present in *A. indica* leaves that accentuate some concrete evidences on their defensive roles against insect pests.

The results indicated that methanol leaf extract exhibited the presence of the highest number of phytochemicals whereas hexane and aqueous extracts demonstrated the lowest. Phenols were present in all leaf extracts while saponins were found in none.

Due to the important role of secondary metabolites, which play in insect-plant interaction that are often involved with plant defense, their bioactivities can be used against stored grain insect pests (Rajashekar et al., 2014). Thus, the presence of secondary metabolite classes may be a useful indicator for both efficacy and potential of phytochemical bioactivity (Gupta et al., 2013). Leaf powders of *A. indica* that were used as bio-insecticides and proved effective on *S. zeamais* were found to contain various constituents after subjecting them to phytochemical screening that were also comparable to those of other researchers who investigated in this regard. However, there were some changes that somewhat not strictly in line with the content of chemical composition of *A. indica* grown in Sri Lanka (Biu et al., 2009;

Krishnaiah et al., 2009; Raphael, 2012; Gupta et al., 2013; Susmitha et al., 2013; Shuaibu et al., 2015). These differences in chemical composition of *A. indica* could be due to the different effects of climatic, seasonal, geographical and environmental factors and also may result from different metabolic pathways in the plant (Guo et al., 2015).

Table 5: Phytochemical constituents of *Azadirachta indica* in, hexane, dichloromethane, ethyl acetate, methanol and aqueous extracts.

Phytochemical Constituent	n-Hexane	Dichloro methane	Ethyl Acetate	Methanol	Aqueous
Alkaloids	+	(-)	(-)	(-)	+
Saponins	(-)	(-)	(-)	(-)	(-)
Flavonoids	(-)	(-)	(-)	(-)	+
Tannins	(-)	(-)	+	+	(-)
Steroids	(-)	+	+	+	(-)
Terpenoids	+	(-)	+	+	(-)
Anthraquinones	(-)	+	+	+	(-)
Glycosides	(-)	+	(-)	+	(-)
Phlobatannins	(-)	+	(-)	(-)	(-)
Coumrins	+	(-)	(-)	(-)	+
Phenols	+	+	+	+	+

(+)=Presence; (-) =Absence

Generally, plants produce a wide array of secondary metabolites that often include insecticidal, repellent, antifeedant or growth retardant properties to control broad spectrum of insect pest damages (Mundi and Alhassan, 2012). Some insects are found to repelled by azadirachtin, isolated from *A. indica* which is a limonoid in the class of terpenoids at the concentrations as low as few parts per million (Defago et al., 2006; Adeyemi, 2011). Not only that, azadirachtin is a natural insecticide that has also been reported to have strong insecticidal, antifeedant and growth disrupting activities towards insect pests, but with very low toxicity to humans and environment (Biu et al., 2009; Krishnaiah et al., 2009). Tannins are toxic to insects because they bind to salivary proteins and digestive enzymes including trypsin and chymotrypsin resulting in protein inactivation, eventually leading to the death of insects. Alkaloids are known as a large class of bitter-tasting nitrogenous compounds that have powerful detrimental effects on animal physiology (Adeyemi, 2011).

Therefore, it can be suggested that compounds found in *A. indica* leaves either individually or in combination may also be responsible for the observed insecticidal and repellent activities of the maize weevils. Additionally, further research on the note of using one or more specific compounds isolated from *A. indica* against the *S. zeamais* might be of great importance.

3.5 Analysis of the volatile organic compounds

With the view of characterising and identifying which volatile organic compounds might be responsible for the fumigation bioactivity demonstrated by the fresh neem leaf powders in the suppression of *S. zeamais*, headspace-solid-phase micro extraction coupled with gas chromatography-mass spectrometry (HS-SPME/GC-MS) was employed. Table 6 presents the area values and the number of volatile organic compounds extracted from headspace of the leaves of *A. indica* using two different fibers. Two fibers of PDMS 30 and PA 85 µm were chosen because both combine the best signal-to-noise ratio with maximum extraction of compounds. At present, this study represents the first ever report on the characterization of volatile compounds from the leaves of *A. indica* by using HS-SPME technique.

Thirty two volatile organic compounds were identified in the leaf of *A. indica* in different proportions with the two SPME fibers. However, higher number of compounds was detected with the medium-polar fiber (PDMS 30 µm) exhibiting comparatively greater area percentages/ relative abundances than with the polar fiber (PA 85 µm).

The major extracted volatile organic compounds were found to be γ-elemene (24.06%), 3,7(11)-eudesmadiene (6.83%), caryophyllene (6.40%), 10s,11s-himachala-3(12),4-diene (6.36%), Neoisolongifolene (3.41%), β-selinene (3.35%), 1,4,7-Cycloundecatriene,1,5,9,9-tetramethyl (3.31%), β-elemene (2.91%), δ-cadinene (2.33%), 2,4-dimethylthiophene (2.31%), longifolene (2.30%) and α-cadinene (2.15%) occupying approximately 65.72% of the total spectrum while other compounds were present in amounts less than 2%.

Chemical class composition of the volatile compounds in the headspace from *A. indica* leaves are illustrated in Table 7. The leaf volatile profile which better extracted from the PDMS 30 µm fiber was dominated by 27 aliphatic molecules (74.97%) followed by 5 aromatic molecules (3.36%), where 21 sesquiterpenoids (71.10%) and 4 monoterpenoids (3.47%) occupied the highest composition of aliphatic molecules. The major sesquiterpenoid contributors in the aliphatic molecular framework were, notably γ-elemene, with the low abundances of 3,7(11)-eudesmadiene, caryophyllene and 10s,11s-himachala-3(12),4-diene constituting over 43% of the fragrant headspace of *A. indica* whilst other constituents present in influential amounts were making a significant contribution in the leaf volatiles of *A. indica*.

In an earlier study, Zeringue and Bhatnagar (1994) had observed the significance of inhibitory effects exerted by the volatile compounds of neem leaves on the fungal growth and aflatoxin production in aflatoxigenic *Aspergillus parasiticus* cultures. They trapped the volatiles on small Tenax glass columns and reported 68 compounds in the GC-MS separation and identification, which were entirely and chemically distinct from those reported in the present study. It must be noted that the above study reported the presence of principal classes of ketones, alcohols, aldehydes, hydrocarbons and miscellaneous compounds while referring 3-hydroxy-2-butanone (19.67%), 2-propanone (12.84%), 2,3-butanediol (9.70%), 1-heptanol (5.43%), 4-pentenal (3.83%) and 2-heptenal (2.60%) as the major contributors in neem leaf volatile composition. Thus, it is apparent that the differences in the nature and composition of volatile organic compounds of neem were the result of the differences in the extraction protocols. On the other hand, Shivashankar et al, (2012) reported the volatile organic compounds present in seed and seed cake of *A. indica* which analyzed by the same extraction protocol as used as in the present study, the HS-SPME/GC-MS. According to their report, most abundant volatile organic components were (Z)-9,7-octadecadienal and palmitic acid comprising 25.47% and 14.97% of the total spectrums in *A. indica* seed and cake respectively.

Table 6: SPME headspace analysis of volatile compounds from leaves of *Azadirachta indica* using PA 85 µm and PDMS 30 µm fibers.

	Volatile Organic Compound[a]	PA 85 µm		PDMS 30 µm	
		RT[b]	Relative Peak Area (%)*	RT[b]	Relative Peak Area (%)*
1	2,4-Dimethylthiophene	ND	-	3.587	2.31
2	3,4-Dimethylthiophene	ND	-	3.801	0.86
3	Bicyclo[4.1.0]hept-2-ene,3,7,7-trimethyl (2-Carene)	9.950	0.79	ND	-
4	4-Carene	ND	-	9.955	1.76
5	4-Methylene-1-(1-methylethyl) cyclohexene (β-Terpinene)	ND	-	10.091	1.71
6	3a,7-Methano-3aH-cylopentacyclooctene, 1,4,5,6,7,8,9,9a-octahydro-1,1,7-trimethyl-[3aR-(3a.alpha., 7.alpha.,9a.beta)] (Clovene)	ND	-	10.319	0.42
7	α-Cubebene	10.499	0.57	ND	-
8	Copanene	ND	-	10.504	1.34
9	Cyclobuta[1,2,3,4]dicyclopentene,decahydro-3a-methyl-6-methylene-1-(1-methylethyl)-, [1S-(1.alpha.,3a.alpha.,3b.beta.,6a.beta.,6b.alpha)] (β-bourbonene)	ND	-	10.632	1.01
10	Cyclohexene,1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)—[1S-(1.alpha.,2.beta.,4.beta.)] (β-Elemene)	10.691	1.35	10.706	2.91
11	Isocaryophillene	ND	-	10.942	0.79
12	Caryophyllene	ND	-	11.129	6.40
13	γ-Elemene	11.233	22.39	11.332	24.06
14	4,7-Methanoazulene,1,2,3,4,5,6,7,8-octahydro-1,4,9,9-tetramethyl-,[1S(1.alpha.,4.alpha.,7.alpha.)] (β-Patchoulene)	11.449	1.02	ND	-
15	Neoisolongifolene	ND	-	11.501	3.41
16	1,4,7-Cycloundecatriene,1,5,9,9-tetramethyl	11.551	0.40	11.590	3.31
17	1H-cyclopropazulene,decahydro-1,1,7-trimethyl-4-methylene-[1aR-(1a.alpha.,4a.beta.,7.alpha.,7a.beta.,7b.alpha)] (Alloaromadedrene)	11.615	0.73	11.813	1.33
18	Naphthalene,1,2,4a,5,8,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1.alpha.,4a.beta.,8a.alpha) (α-Cadinene)	11.993	1.06	12.015	2.15
19	Naphthalene,decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)-,[4aR-(4a.alpha.,7.alpha.,8a.beta)] (β-Selinene)	ND	-	12.084	3.35
20	Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl) (α-Bergamotene)	12.269	0.56	ND	-
21	1H-benzocycloheptene,2,4a,5,6,7,8-hexahydro-3,5,5,9-tetramethyl (β-Himachalene)	ND	-	12.283	1.06
22	Naphthalene,1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(2-methylethyl) (δ-Cadinene)	12.385	0.61	12.407	2.33
23	β –Humelene	12.452	0.42	12.468	0.85
24	10s,11s-Himachala-3(12),4-diene	12.572	2.16	12.609	6.36
25	Naphthalene,1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethyldiene) (3,7(11)-Eudesmadiene)	12.655	2.18	12.701	6.83
26	Longifolene	12.809	0.26	12.878	2.30
27	Azulene,1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1S-(1.alpha.,4.alpha.,7.alpha.)] (α-Guaiene)	12.904	0.17	13.117	0.54
28	5-Allylsulfanyl-1-(4-methoxy-phenyl)-1H-tetrazole	ND	-	13.559	0.40
29	Cadina-1(10),6,8-triene	ND	-	13.657	0.07
30	Cycloisolongifolene,8,9-dehydro	ND	-	13.794	0.10
31	Naphthalene,1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1S-(1.alpha.,7.alpha.,8a.alpha.)] (Valencene)	ND	-	15.146	0.25
32	1-(2-methyoxyphenyl)-2,5-dihydro-1H-pyrrole-2,5-dione	ND	-	15.366	0.12
Total			34.67		78.33

[a]Compounds listed in order of elution, [b]RT=Retention Time, *Data are expressed as percentage of the total peak area, ND=Not Detected

Table 7: Chemical class composition of volatile organic compounds of leaves of *Azadirachta indica*.

Molecular Framework	Class	Percentage (%)	
		PA 85 µm	PDMS 30 µm
Aliphatic	Monoterpenoids	1.35	3.47
	Sesquiterpenoids	33.32	71.10
	Other	-	0.40
Aromatic	Purine nucleosides	-	0.07
	Other	-	3.29
Total		34.67	78.33

Several reports have demonstrated that the plant volatiles produce insect mortality by inhibiting acetylcholinesterase enzyme and bio-fumigants could be neurotoxic based on behavioral symptoms similar to those produced by organophosphates (Rajashekar et al., 2014). In that account, the insecticidal and repellent nature of the leaf powders of *A. indica* manifested by adult insects of *S. zeamais* in both contact and vapor forms may be linked to the main volatile compounds extracted reportedly acting alone or in synergy with other minor constituents (Adjalian et al., 2015).

Monoterpenoids exhibit acute toxic, repellent and antifeedant effects or effects on growth and development or reproduction against target insect species while establishing their biological activity as ovicides, fumigants and contact toxicants on various insect pests. They are typically volatile and rather lipophilic compounds that can penetrate into insects rapidly interfering with their physiological functions (Lee, 1997). Accordingly, β-himachalene, a monoterpenoid has been found to possess insecticidal properties as reported by Singh (2014). Meanwhile sesquiterpenoids have already been better proven for their repellent activities against a wide array of insect pests (Gross and Coats, 2014). Caryophyllene and its derivatives reportedly possess acaricidal, insecticidal, repellent, attractive and antifungal properties. During the laboratory experiments in previous attempts it has been found that this sesquiterpene also mediates the behavioural changes in *Aedes aegypti* leading to a reduction in oviposition, thus suggesting that the compound could be used in controlling the spread of dengue mosquito (Santos da Silva et al., 2015). Additionally, alloaromadendrene, humulene and caryophyllene had been proved to be the the most active toxic sesquiterpenes against the South Asian termites (*Neotermes* spp.) by Messer et al (1960) and Choong and Achmadi (1996). Furthermore, alloaromadendrene is one of the major components in crude resin of *Dipterocarupus* trees that is responsible for the insecticidal properties of this resin against insect pests (Gijsen *et al*., 1995). α-Bergamotene, a volatile defense compound as well as a sesquiterpene performs multiple activities including, repelling herbivorous invaders, decreasing their rates of oviposition and also recruiting their natural predators (Redei, 2008).

Structural characteristics of compounds such as shapes, degree of saturation and types of functional groups influence the insecticidal activity and species-specific susceptibility. The potencies of compounds vary because their chemical properties and structural diversities can elicit different degrees of toxicity (Lee, 1997). Additionally, Grodnitzky and Coats (2002) developed quantitative structure-activity relationship (QSAR) models to explain the chemical basis or importance of electronic properties of compounds responsible for various biological and physiochemical effects, that provide insight into the important regions of the molecules responsible for their insecticidal properties. They further suggested that the compounds in aliphatic framework may also have a different mode of action than the compounds in the aromatic model.

4. Conclusion

Secondary metabolite and biological experiments performed during the current study confirm the eco-chemical phenomena underscoring the insecticidal and repellent properties of neem leaf powders observed against the maize weevil infestations. Though *A. indica* leaves have long been successfully used for the protection of stored grains from insect pest attack in agriculture, it is suggested that further investigations should be directed in line with the above findings towards the quantification and exploration of biological aspects of the principle volatiles produced by neem leaves identified during this study, thereby providing new insights on how *A. indica* leaves showcase their insecticidal and repellent activities, thus making them available to humankind and prospering sustainable development of the nature.

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